THE CHEMICAL COMPOSITION OF THE CRYSTALLINE STYLE AND OF THE GASTRIC SHIELD: WITH SOME NEW OBSERVATIONS ON THE OCCURRENCE OF THE STYLE OXIDASE

C. BERKELEY

(*From the Pacific Biological Station, Nanaimo, B. C.*)

The Crystalline Style

The chemical nature of the crystalline style in the lamellibranchs has interested observers from quite early times. Nelson (1918) gives an excellent summary of the opinions held on the subject up to the time of the publication of his paper and concludes that the style is "a structure of colloid nature resembling mucin."

The view that the constitution of the material of the style resembles that of mucin seems to have originated with Barrois (1889), who showed in the case of *Cardium* that it behaved like a globulin in respect of its solubility and chemical reactions, but also had the property of yielding a reducing substance on hydrolysis with dilute acid. In the absence of carbohydrate substances capable of yielding a sugar on hydrolysis he concluded that this indicated the presence of mucin or chondrin. This conclusion is quoted in some of the early papers in which the constitution of the style of other lamellibranchs is discussed (e.g., List, 1902, in the case of *Mytilus*, and Yonge, 1926, in that of *Ostrea*), but without presentation of any further evidence. More recently reference to mucin is omitted and the substance of the style is regarded as "a protein of a globulin nature" (Yonge, 1931 and 1932). Previously to undertaking the present work I had myself completely confirmed Barrois' observations in the case of a number of species, but his conclusion did not seem justified in the absence of more complete knowledge of the nature of the reducing substances resulting from the hydrolysis of the style material. Any glucoprotein would, for instance, react in the manner indicated and no case is made out for mucin or chondrin in particular.

A good deal of confusion existed as to the respective meanings of these two terms until the matter was clarified by the work of Levene and his co-workers (1922). While formerly substances were classified as chondrins or mucins largely on the basis of their physiological origin and physical condition, provided they had the chemical properties of

proteins coupled with that of yielding a reducing substance on acid hydrolysis, the terms have now a definite chemical significance. In both cases the protein radicle is combined with a complex acid radicle, in that of the chondrins chondroitin sulphuric acid, in that of the mucins mucoitin sulphuric acid. Both of these acids yield on acid hydrolysis glucuronic acid, sulphuric acid, and an acetylated hexosamine, in the former case acetyl-galactosamine, in the latter acetyl-glucosamine. The presence of either mucin or chondrin can, accordingly, be definitely established by the detection of these substances among the products of hydrolysis.

The styles of the following four species of lamellibranch have been examined from this point of view: *Schizothærus nuttalli, Mya arenaria, Ostrea gigas,* and *Saxidomus giganteus.* These four species were selected partly because of their large size and ready availability in sufficient quantity to supply enough material for analysis, and partly because they exemplify two distinct types of crystalline style. Those of the two first-named species are very solid in texture, dissolve only slowly in distilled water, and remain practically intact when the animals are kept under adverse conditions even until death ensues. Those of the two last-named species, on the contrary, are of a much less solid texture, dissolve readily in distilled water, and disappear very rapidly when the animals are kept out of water. In the cases of all three species of "clam" the styles were removed immediately after the animals were dug, dried in a water-oven to constant weight, and stored for analysis. In that of the oyster this procedure could not be followed exactly because it was so frequently found that the style had already begun to soften, or had entirely disappeared, in animals left exposed on the beds by the receding tide. In this case, therefore, the animals were lifted into a large floating perforated tray and left there for some hours until the styles were found fully developed. These were removed immediately after the oyster was taken from the water and stored in alcohol until they could be transferred to the drying oven.

Glucuronic Acid

Glucuronic acid is readily detected in solutions containing relatively little other organic matter by its property of yielding furfurol on boiling with hydrochloric acid. In the presence of any of several polyvalent phenolic substances furfurol gives a series of highly colored compounds. To the solution under test an equal volume of concentrated hydrochloric acid is added and a trace of phloroglucin or orcin; on boiling a violet-red color develops with the former reagent, a violet-blue with the latter.

In application to crystalline style material this test gave inconclusive results since charring of the protein darkened the solutions too much for color-reading to be possible. As used quantitatively, this difficulty is overcome by distilling off the furfurol produced, which can be readily recognised in the distillate by allowing a drop to fall upon aniline-acetate test paper with which it gives a bright cherry red coloration. By this means positive results were obtained from the crystalline styles of each of the four species under consideration. A quantitative comparison was therefore made between them by continuing the distillation in each case until no more furfurol was produced, precipitating it from the distillate with phloroglucin, and weighing as phloroglucide. The detailed procedure was that of the method of Tollens and Kröber for the determination of furfurol derived from pentoses and the results in Table I are calculated from the phloroglucide by Kröber's factor (Browne, 1912).

TABLE I

Species	Weight of Material	Furfurol	Furfurol
			per cent
Schizothaerus nutalli	.5758	.007083	1.23
Mya arenaria	.5939	.007134	1.20
Ostrea gigas	.3890	.004343	1.11
Saxidomus giganteus	.4784	.004653	.97

The four species thus approximate to one another in furfurol-yielding capacity, and hence in the glucuronic acid content, of their crystalline styles, but such difference as occurs indicates a consistently lower content in the more soluble styles.

GLUCOSAMINE

The method described by Elson and Morgan (1933) for the determination of glucosamine and chondrosamine was applied qualitatively to all the acid residues from which the furfurol had been distilled in the determinations of glucuronic acid described in the previous paragraph. If mucin or chondrin were present in the crystalline style, it was anticipated that the corresponding amino sugar would remain in the solutions as hydrochloride after boiling with hydrochloric acid. For this reason the method of Elson and Morgan for glucosamine and chondrosamine was employed rather than the modification for the estimation of the acetylated compounds described in their later paper (1934). The solutions were made up to equal volume and 5 cc. of each was neutralised, filtered, and submitted to the test. In each case a positive reaction was

obtained while a blank test with the reagents remained entirely colorless.

A comparison was made quantitatively between the style of *Ostrea gigas* and that of *Schizothœrus nutalli*. The residual solutions from the furfurol distillations were again used for this purpose. It was necessary in the first place to ensure that all the amine present had been set free from combination. To this end 5 cc. of each of the solutions was evaporated to small volume and the residue taken up with 5 cc. concentrated HCl and heated in a boiling water bath for two hours under a reflux condenser. The resulting solutions were neutralised and filtered, whereby only slightly colored filtrates were obtained, and the depth of color developed in Elson and Morgan's test compared with that from 5 cc. of the original solution after neutralisation and filtration. All the filtrates were made up to like volume before applying the test. In neither case could any alteration in the depth of color be detected as a result of the further heating with HCl. It was concluded, therefore, that the hydrolysis had already reached a maximum in respect of the amine constituent in the residues from the furfurol determinations. Comparison was accordingly made between the solutions without further hydrolysis.

The weights of *Ostrea* and *Schizothœrus* material respectively taken for the furfurol determinations were almost exactly in the ratio of 1 : 1.5 (see table), so that volumes of the residual solutions taken in this ratio represented equal weights of material. Accordingly 5 cc. of the solution obtained from *Schizothœrus* and 7.5 of that from *Ostrea* were withdrawn, neutralised, filtered, the filtrates made up to equal volume, and tested. Very little difference could be detected in the colors developed on standing, indicating a practical equality in the glucosamine content in the styles of the two species.

Sulphuric and Acetic Acids

A series of experiments parallel to those described in the foregoing paragraph was carried out with the residues from the furfurol distillations for the detection and attempted estimation of sulphuric acid, substituting precipitation with barium chloride in acid solution for the Elson and Morgan test.

All the solutions gave positive reactions, but the amounts of precipitate obtained from 5-cc. samples were very small and, in the case of *Ostrea*, only just detectable. In 25-cc. samples the precipitates were still too small to determine gravimetrically and further hydrolysis led to no perceptible increase in the amounts. Attempts at quantitative determination were therefore abandoned, but rough estimates were made

by eye of the relative amounts of precipitate obtained from aliquots of the four solutions representing equal weights of material. These pointed fairly definitely to the presence of more sulphuric acid in the styles of *Schizothærus* and *Mya* than in those of *Saxidomus* and *Ostrea,* the last named being markedly the poorest in that constituent.

No attempt was made to detect the acetic acid radicle in the residues from the furfurol determinations. There is no method by which the trace whose occurrence would be anticipated from the small amount of material taken for analysis could be recognised with certainty in the presence of the relatively large amount of hydrochloric acid.

Discussion

It has thus been shown that the crystalline styles of the four species investigated contain the essential constituents of the acids characteristic of mucin and chondrin. Elson and Morgan's test does not serve to differentiate glucosamine and chondrosamine, the respective amino sugars of these two acids, so that a definite conclusion cannot be drawn as to which of them is present in the style. A great deal more material than could readily be obtained would be necessary for this purpose, but, judging by its solubility and ease of hydrolysis with acid, it is extremely probable that mucin rather than chondrin is involved.

There seem to be some slight differences in the composition of the styles of the four species investigated, those of *Schizothærus* and *Ostrea,* for instance, agreeing very closely in their content of glucosamine, but differing in that of glucuronic and sulphuric acids. This suggests that the material consists of a mixture of mucin and a glucoprotein having glucosamine as its carbohydrate constituent; or, in other words, of a glucosamine glucoprotein a variable part of which is united with mucoitin sulphuric acid to form mucin.

If this be so, the differences in solubility of the styles in various species may be associated with their content of mucin. Yonge (1932) has shown that the readiness with which the style disappears when the living animals are exposed to adverse conditions can be correlated with the presence or absence of a separate style-sac; those species in which this is not present losing their styles more readily, when the secretion of the style material is inhibited, owing to their exposure to the solvent action of the digestive fluid of the stomach and intestine. While this is undoubtedly the case, it does not seem to explain the variation in solubility of the styles of various species when removed from the animal and placed in distilled water. *Schizothærus* affords an instance of an animal with a complete style-sac, *Ostrea* of one with none. In the former case, the style persists after long periods of adverse conditions, in the latter

it disappears very rapidly; but, as has been pointed out, the style of *Schizothærus* dissolves very slowly in distilled water and that of *Ostrea* very quickly and the former appears to have a greater amount of its glucoprotein in mucin combination. Chemical composition, therefore, may also be a factor in the solubility of the style material and influence its rate of disappearance in adverse circumstances under the action of the digestive fluid.

The Gastric Shield

The only statement I have been able to trace in connection with the chemical composition of the gastric shield is that of Nelson (1918). "From its consistency and action toward common reagents" this author concludes "it is probably in the nature of chondrin." This conclusion is quoted by Yonge (1926) in support of his view that "the gastric shield is not a secretion, but is formed by the fusion of cilia, originally in response to the irritation caused by the head of the style."

Schizothærus nutalli has a large gastric shield readily separable from the stomach wall and it was obtainable in fairly large quantity. It was therefore taken for investigation.

It was found at an early stage that the shield could be boiled with 60 per cent potash without dissolving or undergoing appreciable decomposition, the structure preserving its characteristic shape even after prolonged boiling and standing. This rendered a chondrin-like composition extremely doubtful since all chondroid substances hitherto investigated have been found to hydrolyse on alkaline digestion. Chitin seemed to be the compound having the property of resisting attack by strong alkali most likely to occur in the situation in which the gastric shield is found and the material was accordingly examined from this standpoint. After boiling for some time with 60 per cent potash, it was thoroughly washed and dried. It was then found to give the reaction characteristic of chitin with sulphuric acid and iodine and to dissolve readily in boiling concentrated hydrochloric acid. The solution in hydrochloric acid gave off ammonia on making alkaline and boiling, it had a strong reducing action on Fehling's solution, and gave a crystalline osazone with phenylhydrazine. All these tests pointed to chitin. Finally some of the material was submitted to the treatment described in the earlier part of this paper for identifying the constituents of the crystalline style. On boiling with hydrochloric acid and distilling, no furfurol could be detected in the distillate. The residual solution gave no reaction for sulphuric acid, but the presence of glucosamine was shown by the test of Elson and Morgan. Chitin consists of polymerised acetyl glucosamine. There is thus no doubt that the substance

of which the gastric shield is composed is chitin and that it contains no chondrin-like constituent.

Nelson's (1918) observation that the gastric shield gives the biuret test and breaks up in the process under the action of the strong alkali employed is probably to be attributed to an incomplete separation of the adjacent tissues and of the substance of the crystalline style which is always more or less adherent to it.

As bearing on Yonge's (1926) view of the origin of the gastric shield, it is of interest to speculate whether the shield may not be derived from the substance of the style itself. From the chemical standpoint it does not seem impossible that chitin could be formed from mucin by the elimination of glucuronic and sulphuric acids from mucoitin-sulphuric acid and polymerisation of the remaining acetyl glucosamine. The related compositions of mucin and chitin have previously been discussed from this point of view by Matthews (1916), who draws attention to the occurrence in large quantity of mucin in the skin of the lower vertebrates and of chitin in the hard covering of the arthropods and considers that the " facts are of interest in the light of the theory of Gaskell and Patten that the arthropods were the ancestors of the vertebrates."

The Style Oxidase

The occurrence of an oxidase system in the crystalline style of the lamellibranchs has hitherto been recorded in the cases of thirteen species which I have enumerated in a previous paper (Berkeley, 1933). Two more, *Panope generosa* and *Pholadidea penita*, may now be added to the list. I have recently detected considerable oxidase activity in solutions of the styles of both of these. The oxidase has now been observed in a sufficiently large range of families to render it extremely probable that its occurrence is general throughout the lamellibranchs.

The presence of the oxidase has not, hitherto, been recorded in the style of any species of gastropod. Unlike the case of the lamellibranchs, the style is by no means of general occurrence in the gastropods. Its presence has, in fact, been observed in only a very limited number of genera. Yonge (1932) gives a complete list of these and points out that the style occurs only in such gastropods as are herbivores and which " either by ciliary currents or by a radula, pass a continuous supply of finely divided food to the stomach."

The only large marine species among them which I have been able to obtain in a living condition is *Crepidula fornicata*. This species is common in the oyster beds at Olympia, Washington, whence a number of live specimens were recently obtained. On arrival, some 24 hours after being taken from the beds, none of those opened had any crystal-

line styles. After being kept in the sea for some days, a large number died, but all the survivors contained styles and supplied sufficient material to enable me to carry out a series of oxidase tests. The results of these were quite definitely positive.

Summary

The crystalline styles of four species of lamellibranch have been examined chemically to determine whether the material is entirely protein in nature or contains mucin or chondrin.

The material yields on acid hydrolysis, glucuronic acid, sulphuric acid and a hexosamine, in addition to protein. It therefore contains all the essential constituents of mucin or chondrin. The solubility and ease of hydrolysis of the material suggest that mucin rather than chondrin is involved.

The composition of the styles is not quantitatively identical in the four species examined, but varies in such a way as to suggest that the less readily soluble styles contain the larger quantity of mucin.

The material of which the gastric shield is composed is found to be chitin.

The oxidase system previously recorded in the styles of a number of lamellibranchs is now shown to occur in two more species. It is also found to be present in that of the gastropod, *Crepidula fornicata*.

REFERENCES

Barrois, T., 1889. Le Stylet Cristallin des Lamellibranches. *Rev. Biol. Nord du France*, 3, 4: 124, 161, 263; 5: 209, 299, 351.

Berkeley, C., 1933. The Oxidase and Dehydrogenase Systems of the Crystalline Style of Mollusca. *Biochem. Jour.*, 27: 1357.

Browne, C. A., 1912. Handbook of Sugar Analysis. First Edition, p. 450.

Elson, L. A., and W. T. J. Morgan, 1933. A Colorimetric Method for the Determination of Glucosamine and Chondrosamine. *Biochem. Jour.*, 27: 1824.

Levene, P. A., 1922. Hexosamines, Their Derivatives, and Mucins and Mucoids. *Mons. Rockefeller Inst. for Med. Res.*, No. 18.

List, T., 1902. Die Mytiliden. Fauna und Flora des Golfes von Neapel, 27, p. 274.

Matthews, A. P., 1916. Physiological Chemistry. Second Edition, p. 324.

Morgan, W. T. J., and L. A. Elson, 1934. A Colorimetric Method for the Determination of N-Acetyl-glucosamine and N-Acetyl-chondrosamine. *Biochem. J.*, 28: 988.

Nelson, T. C., 1918. On the Origin, Nature, and Function of the Crystalline Style of Lamellibranchs. *Jour. Morph.*, 31: 53.

Yonge, C. M., 1926. Structure and Physiology of the Organs of Feeding and Digestion in Ostrea edulis. *Jour. Mar. Biol. Ass.*, 14: 295.

Yonge, C. M., 1931. Digestive Processes in Marine Invertebrates and Fishes. *Jour. Con. Perm. Intern. Explor. Mer.*, 6: 187.

Yonge, C. M., 1932. Notes on Feeding and Digestion in Pterocera and Vermetus. With a Discussion on the Occurrence of the Crystalline Style in the Gastropoda. *Rep. Great Barrier Reef Exped.*, vol. 1, No. 10.